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(54) Title: INTERFERON-γ INDUCING FACTOR IN NEUROENDOCRINE CELLS

(57) Abstract

The present invention relates to isolated DNA molecules encoding interferon-γ inducing factors, interleukin-18 and interleukin-18α, from rat. The invention also provides the rat interferon-γ inducing factor proteins or polypeptides identified as interleukin-18 and interleukin-18α. The use of these materials is also disclosed.

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INTERFERON-γ INDUCING FACTOR IN NEUROENDOCRINE CELLS

The present application claims the benefit of U.S. Provisional Patent Application Serial No. 60/025,141, filed September 9, 1996, and U.S. Provisional Patent Application Serial No. 60/043,087, filed April 8, 1997.

FIELD OF THE INVENTION

The present invention relates to the interferon-γ inducing factors, interleukin-18 and interleukin-18α, from rat and the DNA molecules encoding these interferon-γ inducing factors. The invention also provides for utilizing these interferon-γ inducing factors.

BACKGROUND OF THE INVENTION

A growing family of regulatory proteins that deliver signals between cells of the immune system has been identified. These regulatory molecules are known as cytokines. Many of the cytokines have been found to control the growth, development and biological activities of cells of the hematopoietic and immune systems. These regulatory molecules include all of the colony-stimulating factors (GM-CSF, G-CSF, M-CSF, and multi CSF or interleukin-3), the interleukins (IL-1 through IL-11), the interferons (alpha, beta and gamma), the tumor necrosis factors (alpha and beta) and leukemia inhibitory factor (LIF). These cytokines exhibit a wide range of biologic activities with target cells from bone marrow, peripheral blood, fetal liver, and other lymphoid or hematopoietic organs. See, e.g., G. Wong and S. Clark, Immunology Today, 9(5):137 (1988).

Interferon is a specialized protein which is produced by infected cells. Interferon acts to confine the infection to the already infected cells. The two main effects of interferon are inhibitory: it prevents virus replication and inhibits cell growth. There are at least

three types of interferon, alpha, beta, and gamma. Gamma interferon ("γ-INF") is produced by mitogen- or antigen-stimulated T lymphocytes. It differs from alpha and beta interferon in that it is labile at a pH of 2 and in that it has a more pronounced anticellular than antiviral activity.

Interferon-γ inducing factor ("IGIF") is a newly identified cytokine first isolated from Kupffer cells of mice injected with *Propionibacterium acnes* and challenged with lipopolysaccharide (LPS) to induce toxic shock (Nakamura, K. et al., Infect. Immun., 61:64-70 (1993); Okamura, H. et al., Infect. Immun., 63:3966-3972 (1995); and Okamura, H. et al., Nature, 378:88-91 (1995)). The names of interleukin-1γ ("IL-1γ") and of interleukin-18 (IL-18) also have been proposed on the basis of homology with the structure of human interleukins-1 (Bazan, J.F. et al., Nature, 379:591 (1996)) and of IGIF's peculiar activity (Ushio, S. et al., J. Immunol., 156:4274-4279 (1996)), respectfully. Mouse IGIF has been shown to induce interferon-γ production by Th1 cells, to stimulate natural killer cell (NK) proliferation, and to mediate inflammatory tissue damage (Nakamura, K. et al., Infect. Immun., 61:64-70 (1993)). Further studies with human IGIF (Ushio, S. et al., J. Immunol., 156:4274-4279 (1996)) showed this cytokine increased the production of granulocyte macrophage-colony-stimulating factor and decreased that of interleukin-10 ("IL-10"). Taken together, these data imply that IGIF is a potent cytokine involved in the cell-mediated immune response and might serve as an antimicrobial and antitumor agent.

A growing number of findings support the idea that the nervous, endocrine, and immune systems form an integrated network of fundamental importance for the homeostasis of the organism (Blalock, J., Immunol. Today, 13:504-511 (1994)). Communication among these systems is possible because they share ligands and receptors previously

thought to be tissue-specific. Indeed, stress perceived by the nervous and endocrine systems can induce a heightened immune response.

Stress is defined broadly as the result produced in an organism when it is acted upon by forces that disrupt equilibrium or produce strain. Tabor's Medical Dictionary, F.A. Davis Co., Philadelphia (1982). It is generally believed that biological organisms require a certain amount of stress to maintain their well-being. However, when stress occurs in quantities that the system cannot handle, it produces pathological changes. See, Tabor's Medical Dictionary, F.A. Davis Co., Philadelphia (1982).

Due to the broad definition of stress, it is difficult to quantitate. However, an effective means of quantitating stress would be useful for maintaining stress levels in a safe range. For example, when stress levels approach dangerous levels, steps can be taken to reduce or avoid stress before pathological changes result. The present invention is directed to addressing this need.

SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acid molecules which encode rat interleukin-18 protein or polypeptide and rat interleukin-18α protein or polypeptide.

Also disclosed is the isolated rat interleukin-18 protein or polypeptide and rat interleukin-18α protein or polypeptide.

A further embodiment of the invention is a method for detecting interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide in a sample of tissue or body fluids. An antibody or binding portion thereof is contacted with the sample. The detection of any reaction, using an assay system, indicates that an interleukin-18

protein or polypeptide or an interleukin-18α protein or polypeptide is present in the sample.

The invention also provides a method for detecting an interleukin-18 protein or polypeptide or an interleukin-18α protein or polypeptide using a DNA molecule which encodes interleukin-18 or interleukin-18α as a probe in a nucleic acid hybridization assay or a gene amplification detection procedure. A sample is contacted with the probe. The detection of any reaction, using an assay system, indicates that a nucleic acid encoding interleukin-18 or interleukin-18α or a fragment thereof is present in the sample.

The invention further provides a method for quantitating stress in a mammal. An antibody or binding portion thereof specific to interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide is contacted with a sample of tissue or body fluid of the mammal. The amount of interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide present in the sample, which is indicative of the stress in the mammal, is measured using an assay system.

Another embodiment of the invention is a method for quantitating stress in a mammal using a DNA molecule which encodes interleukin-18 or interleukin-18α as a probe in a nucleic acid hybridization assay. Tissue or body fluid of the mammal is contacted with the probe, and the amount of interleukin-18 mRNA or interleukin-18α mRNA present in the sample, which is indicative of the stress in the mammal, is measured using an assay system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides dark field photoemulsion autoradiograms showing IGIF mRNA in the adrenal and pituitary glands by *in situ* hybridization. The adrenal

glands were analyzed in the control animal (A), after exposure of the animal at 4°C for 4 h *(B)*, 24 h after cold stress was given *(C)*, 4 h after vehicle *(D)* and reserpine treatment *(E)*. IGIF mRNA was also detected in neurohypophysis (F). Med., the adrenal medulla; *zr*, zona reticularis; *zf*, zona fasciculata; *zg*, zona glomerulosa; *Ah*, adenohypophysis; and *Nh*, neurohypophys. *Bar* is 150 μm is *A-E* and 300 μm in *F*.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides an isolated nucleic acid molecule encoding a rat interferon-γ inducing factor ("IGIF"). This nucleic acid molecule can be a DNA molecule comprising the nucleotide sequence corresponding to SEQ. ID. No. 1 as follows:

ATGGCTGCCA TGTCAGAAGA AGGCTCTTGT GTCAACTTCA AAGAAATGAT 50
GTTTATTGAC AACACACTTT ACCTTATACC TGAAGATAAT GGAGACTTGG 100
AATCAGACCA CTTTGGCAGA CTTCACTGTA CAACCGCAGT AATACGGAGC 150
ATAAATGACC AAGTTCTCTT CGTTGACAAA AGAAACCCGC CTGTGTTCGA 200
GGACATGCCT GATATCGACC GAACAGCCAA CGAATCCCAG ACCAGACTGA 250
TAATATATAT GTACAAAGAT AGTGAAGTAA GAGGACTGGC TGTGACCCTA 300
TCTGTGAAGG ATGGAAGGAT GTCTACCCTC TCCTGTAAAA ACAAAATCAT 350
TTCCTTTGAG GAAATGAATC CACCTGAAAA TATTGATGAT ATAAAAAGTG 400
ATCTCATATT CTTTCAGAAA CGTGTGCCAG GACACAACAA AATGGAATTT 450
GAATCTTCCC TGTATGAAGG ACACTTTCTA GCTTGCCAAA AGGAAGATGA 500
TGCTTTCAAA CTCGTTTTGA AAAGGAAGGA TGAAAATGGG GATAAATCTG 550
TAATGTTCAC TCTTACTAAC TTACATCAAA GTTAGGTATT AAGGTTTCTG 600
TATTCCAGAA AGACGATTAG TATACACGAG CCTTATGATA ACCTACTCTG 650
TATTTCTATG ACAAAATACC TGAGGCCGCA TGATTTATAG AGTAAACAAG 700
CTTGATTGCC CAAAAAAAAA AA 750

The above DNA molecule encodes for a polypeptide having a molecular weight of about 20 to 24 kilodaltons,

preferably 22.3 kilodaltons. The first 36 amino acids of the polypeptide of SEQ. ID. No. 2 are a leader peptide, which is removed *in vivo*. The processed polypeptide lacking a leader sequence has a molecular weight of about 16 to 20 kilodaltons, preferably 18.3 kilodaltons. The amino acid sequence, deduced from the nucleotide sequence corresponding to SEQ. ID. No. 1, represents a form of rat IGIF, which is also referred to as interleukin-1γ or interleukin-18 ("IL-18"). It is predicted that this protein or polypeptide has the deduced amino acid sequence corresponding to SEQ. ID. No. 2 as follows:

Met Ala Ala Met Ser Glu Glu Gly Ser Cys Val Asn Phe Lys
1 5 10
Glu Met Met Phe Ile Asp Asn Thr Leu Tyr Leu Ile Pro Glu
15 20 25
Asp Asn Gly Asp Leu Glu Ser Asp His Phe Gly Arg Leu His
30 35 40
Cys Thr Thr Ala Val Ile Arg Ser Ile Asn Asp Gln Val Leu
45 50 55
Phe Val Asp Lys Arg Asn Pro Pro Val Phe Glu Asp Met Pro
60 65 70
Asp Ile Asp Arg Thr Ala Asn Glu Ser Gln Thr Arg Leu Ile
75 80
Ile Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val
85 90 95
Thr Leu Ser Val Lys Asp Gly Arg Met Ser Thr Leu Ser Cys
100 105 110
Lys Asn Lys Ile Ile Ser Phe Glu Glu Met Asn Pro Pro Glu
115 120 125
Asn Ile Asp Asp Ile Lys Ser Asp Leu Ile Phe Phe Gln Lys
130 135 140
Arg Val Pro Gly His Asn Lys Met Glu Phe Glu Ser Ser Leu
145 150

Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu Asp Asp Ala
155 160 165
Phe Lys Leu Val Leu Lys Arg Lys Asp Glu Asn Gly Asp Lys
170 175 180
Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
185 190

In the preceding nucleotide sequence (i.e. SEQ. ID. No. 1), the underlining signifies a deletion not found in a second cDNA encoding rat IGIF. The shorter isoform lacks a fragment of 57 bases, a probable exon, and the corresponding 19 amino acids in the predicted peptide. This second, lower abundant isoform is called interleukin-18α ("IL-18α"). The DNA molecule which encodes IL-18α has the following nucleotide sequence (SEQ. ID. No. 3):

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ATGGCTGCCA TGTCAGAAGA AGGCTCTTGT GTCAACTTCA AAGAAATGAT   50
GTTTATTGAC AACACACTTT ACCTTATACC TGAAGATAAT GGAGACTTGG  100
AATCAGACCA CTTTGGCAGA CTTCACTGTA CAACCGCAGT AATACGGAGC  150
ATAAATGACC AAGTTCTCTT CGTTGACAAA AGAAACCCGC CTGTGTTCGA  200
GGACATGCCT GATATCGACC GAACAGCCAA CGAATCCCAG ACCAGACTGA  250
TAATATATAT GTACAAAGAT AGTGAAGTAA GAGGACTGGC TGTGACCCTA  300
TCTGTGAAGG ATGGAAGGAT GTCTACCCTC TCCTGTAAAA ACAAAATCAT  350
TTCCTTTGAG AAACGTGTGC CAGGACACAA CAAAATGGAA TTTGAATCTT  400
CCCTGTATGA AGGACACTTT CTAGCTTGCC AAAAGGAAGA TGATGCTTTC  450
AAACTCGTTT TGAAAAGGAA GGATGAAAAT GGGGATAAAT CTGTAATGTT  500
CACTCTTACT AACTTACATC AAAGTTAGGT ATTAAGGTTT CTGTATTCCA  550
GAAAGACGAT TAGTATACAC GAGCCTTATG ATAACCTACT CTGTATTTCT  600
ATGACAAAAT ACCTGAGGCC GCATGATTTA TAGAGTAAAC AAGCTTGATT  650
GCCCAAAAAA AAAAA
```

The nucleotide sequence corresponding to SEQ. ID. No. 3 encodes the following amino acid sequence (SEQ. ID. No. 4):

Met Ala Ala Met Ser Glu Glu Gly Ser Cys Val Asn Phe Lys
1 5 10
Glu Met Met Phe Ile Asp Asn Thr Leu Tyr Leu Ile Pro Glu
15 20 25
Asp Asn Gly Asp Leu Glu Ser Asp His Phe Gly Arg Leu His
30 35 40
Cys Thr Thr Ala Val Ile Arg Ser Ile Asn Asp Gln Val Leu
45 50 55
Phe Val Asp Lys Arg Asn Pro Pro Val Phe Glu Asp Met Pro
60 65 70
Asp Ile Asp Arg Thr Ala Asn Glu Ser Gln Thr Arg Leu Ile
75 80
Ile Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val
85 90 95
Thr Leu Ser Val Lys Asp Gly Arg Met Ser Thr Leu Ser Cys
100 105 110
Lys Asn Lys Ile Ile Ser Phe Glu Lys Arg Val Pro Gly His
115 120 125
Asn Lys Met Glu Phe Glu Ser Ser Leu Tyr Glu Gly His Phe
130 135 140
Leu Ala Cys Gln Lys Glu Asp Asp Ala Phe Lys Leu Val Leu
145 150
Lys Arg Lys Asp Glu Asn Gly Asp Lys Ser Val Met Phe Thr
155 160 165
Leu Thr Asn Leu His Gln Ser
170 175

This protein or polypeptide has a molecular weight of 18-22 kilodaltons, preferably 20 kilodaltons. As with interleukin-18, the first 36 amino acids of interleukin-18α are a leader peptide, which is removed *in vivo*. The processed polypeptide lacking a leader sequence has a molecular weight of about 16 to 20 kilodaltons, preferably 18.3 kilodaltons.

The nucleic acid molecule can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), genomic or

recombinant, biologically isolated or synthetic. The invention encompasses the DNA sequences as well as their complements. The DNA molecule can be a cDNA molecule, which is a DNA copy of a messenger RNA (mRNA) encoding the IGIF protein. A suitable RNA molecule is mRNA.

Suitable nucleic acid molecules include those nucleic acid molecules encoding an IGIF protein and having a nucleotide sequence which is at least 95% homologous to the nucleotide sequence of wild-type rat interleukin-18 or interleukin-18α (collectively referred to as "IGIF") (as shown in SEQ ID No. 1 or SEQ ID No. 3.

While the nucleotide sequence is at least 95% homologous, nucleotide identity is not required. As should be readily apparent to those skilled in the art, various nucleotide substitutions are possible which are silent mutations (i.e. the amino acid encoded by the particular codon does not change). It is also possible to substitute a nucleotide which alters the amino acid encoded by a particular codon, where the amino acid substituted is a conservative substitution (i.e. amino acid "homology" is conserved). It is also possible to have minor nucleotide and/or amino acid additions, deletions, and/or substitutions in the wild-type IGIF nucleotide and/or amino acid sequences which do not alter the function of the resulting IGIF.

Alternatively, suitable DNA sequences may be identified by hybridization to SEQ. ID. Nos. 1 or 3 under stringent conditions. In particular, suitable sequences would hybridize to SEQ. ID. Nos. 1 or 3 under highly stringent conditions where a nucleic acid encoding mouse IGIF would not hybridize. For example, sequences can be isolated that hybridize to a DNA molecule comprising a nucleotide sequence of 50 continuous bases of SEQ. ID. Nos. 1 or 3 under stringent conditions characterized by a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of 37°C and remaining bound when

subject to washing with the SSC buffer at 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M saline/0.09M SSC buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2x SSC buffer at 42°C.

The DNA molecule encoding IGIF polypeptides or proteins can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series

(see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may

not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *rec*A promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5′ to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding IGIF polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but

are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The invention also provides an antisense nucleic acid molecule that is complementary to the mRNA encoding the IGIF, or a fragment thereof capable of hybridizing under stringent conditions to the mRNA. The antisense nucleic acid molecule is ribonucleic acid. This antisense molecule can base-pair with the mRNA, preventing translation of the mRNA into protein.

The invention further provides an isolated fragment of the nucleic acid molecule encoding IGIF. Nucleic acid molecules encoding IGIF proteins, and fragments of the nucleic acid molecules, are thus provided.

Each of the nucleic acid molecules, fragments thereof, antisense nucleic acid molecules, and fragments thereof, can be expressed in suitable host cells using conventional techniques. Such techniques may involve the use of expression vectors which comprise the nucleic acid molecules, fragments thereof, antisense nucleic acid molecules, or fragments thereof. These expression vectors can then be used to transform suitable host cells.

Host cells transformed with nucleic acid molecules encoding IGIF can be used to produce IGIF proteins (or cells transformed with the fragments can be used to produce fragments of the IGIF proteins). Alternatively, the fragments or full-length IGIF proteins can be produced synthetically using the sequence information of the IGIF proteins and fragments. In host cells transformed with the antisense nucleic acid molecules, or fragments thereof, the antisense nucleic acid molecules or fragments thereof will block translation of IGIF. Accordingly, in host cells transformed with the antisense nucleic acid molecules or fragments thereof, the expression of IGIF is decreased.

The protein or polypeptide of the present invention is preferably produced in purified form

(preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is secreted into the growth medium of recombinant host cells. The first 36 amino acids are a leader sequence which is cleaved *in vivo*. Alternatively, the protein or polypeptide of the present invention is produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC. Isolated IGIF protein may be combined with a compatible carrier.

Fragments of the above polypeptide or protein are also encompassed by the present invention. Suitable fragments can be produced by several means. In the first, subclones of the gene encoding the protein of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for IGIF.

As an alternative, fragments of IGIF protein can be produced by digestion of an IGIF protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave IGIF proteins at different sites based on the amino acid sequence of an IGIF protein. Some of the fragments that result from proteolysis may be active IGIF.

In another approach, based on knowledge of the primary structure of the protein, fragments of the IGIF protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of an IGIF peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the IGIF protein being produced. Alternatively, subjecting a full length IGIF protein to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure, and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

Antibodies can also be raised to each of the IGIF proteins, and to the isolated fragments thereof. Antibodies of the subject invention include polyclonal antibodies and monoclonal antibodies which are specific for IGIF or isolated fragments thereof. In addition to utilizing whole antibodies, the present invention encompasses use of binding portions of such antibodies. Such binding portions include Fab fragments, $F(ab')_2$ fragments, and Fv fragments. Such antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in J. Goding, <u>Monoclonal Antibodies: Principles and</u>

Practice, pp. 98-118 (N.Y. Academic press 1983), which is hereby incorporated by reference. These antibodies or fragments thereof can thus be used to detect the presence of an IGIF protein in a sample (or to detect the presence of a fragment of IGIF), by contacting the sample with the antibody or fragment thereof. The antibody or fragment thereof binds to an IGIF protein or fragment thereof present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting the presence of the IGIF protein or fragment thereof in the sample.

Gene amplification can also be used to obtain very high levels of expression of transfected gene. When cell cultures are treated with methotrexate ("Mtx"), an inhibitor of a critical metabolic enzyme, dihydrofolate reductase ("DHFR"), most cells die, but eventually some Mtx-resistant cells grow up. A gene to be expressed in cells is cotransfected with a cloned DHFR gene, and the transfected cells are subjected to selection with a low concentration of Mtx. Resistant cells that have taken up the DHFR gene (and, in most cases, the cotransfected gene) multiply. Increasing the concentration of Mtx in the growth medium in small steps generates populations of cells that have progressively amplified the DHFR gene, together with linked DNA. Although this process takes several months, the resulting cell cultures capable of growing in the highest Mtx concentrations will have stably amplified the DNA encompassing the DHFR gene a hundredfold or more, leading to significant elevation of the expression of the cotransfected gene.

Once the nucleic acid molecule encoding IGIF has been inserted into a host cell, with or without the use of an intermediate expression vector, the host cell can be used to produce IGIF protein by culturing the cell under conditions suitable for translation of the DNA molecule, thereby expressing the IGIF protein. The IGIF protein can

then be recovered from the cell. Generally, the IGIF protein of the present invention is produced in purified form by conventional techniques, such as by secretion into the growth medium of recombinant *E. coli*. To isolate the protein, the *E. coli* host cell carrying a recombinant plasmid is propagated, homogenized, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The invention also provides a method of producing a rat interferon-γ inducing factor. The method involves transforming a cell with DNA encoding interleukin-18 or interleukin-18α, and causing the cell to express said nucleic acid molecule and to produce a rat interferon-γ inducing factor. Full length cDNA clones for IGIF can be inserted into appropriate expression vectors and used in gene transfer experiments to set up transient and stable expression systems in mammalian cell lines.

a) Transient Expression Systems:

A transient expression system (Gorman, C., "DNA Cloning; A Practical Approach", Oxford, IRL Press, 143-190 (1985), which is hereby incorporated by reference), can be used due to the rapidity possible with this assay. Messenger RNA and protein synthesis can be analyzed within 48 hours after the introduction of DNA. Large quantities of specific mRNA (as much as 1% of total cellular mRNA) frequently can be expressed. In contrast, construction of stable transformed cell lines is lengthy, and the levels of expression of mRNA are frequently below that obtained with transient systems.

IGIF can be introduced into SV40 expression vectors (e.g., pSV2) into COS-7 cells, using the calcium phosphate (Wigler, M. et al., Cell, 14:725 (1978), which is hereby incorporated by reference) or DEAE-Dextran (Lopata, M.A. et al., Nucl. Acids Res., 12:5707 (1984), which is hereby incorporated by reference) transfection procedures. Previous studies with transfection of n-Acetyl choline receptor (nAChR) genes found significant transcription of AChR mRNA to levels of about 1% of total mRNA in transfected cells. (Claudio, T. et al., Science, 238:1688-1694 (1987); Claudio, T. et al., "Cloning and Transfer of Acetylcholine Receptor Genes In: Molecular Neurobiology: A Short Course," McKay, R.D., Ed., Bethesda, Neuroscience Society, 22-27 (1984), which are hereby incorporated by reference). Transfected cells can be tested for their level of expression of IGIF mRNA species. Other common cell types can also be tested for transient transfection, e.g., CHO cells, mouse fibroblasts (L cells, 3T3 or 3T6 cells), HeLa cells, neuroblastoma, L6 muscle cells, etc. Studies with primate cells utilize SV40 expression vectors, whereas studies with other cells utilize Rous Sarcoma Virus vectors (i.e., pRSV), which is the most ubiquitous promoter for efficient transient expression.

b) Stable Expression Systems:

Stable cell lines (Claudio, T. et al., Science, 238:1688-1694 (1987); Claudio, T. et al., "Cloning and Transfer of Acetylcholine Receptor Genes In: Molecular Neurobiology: A Short Course," McKay, R.D., Ed., Bethesda, Neuroscience Society, 22-27 (1984), which are hereby incorporated by reference) with transfected IGIF cDNAs can be established for detailed pharmacological and biochemical characterization. Transient expression experiments can be used to determine which viral expression vectors are most efficient in particular cell types. For instance (Gorman, C., "DNA Cloning; A Practical Approach," Oxford, IRL Press,

143-190 (1985), which is hereby incorporated by reference) cells can be co-transfected with IGIF in a pSV or pRSV vector, along with a dominant selectable marker such as gpt or neoR (i.e., in vectors pRSV-gpt or pRSV-neo). The cells will be subcultured into a selective medium two days following transfection, and then once every 4-5 days thereafter until discrete colonies can be seen on transfected plates, requiring 1-2 months to establish stable cell lines. Cells selected by dominant marker can then be tested for expression of IGIF as well.

A further embodiment of the invention is a method for detecting interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide in a sample of tissue or body fluids. The antibodies can be used to distinguish and identify isoforms of interleukin-18 in rat or other species. An antibody or binding portion thereof is contacted with the sample. The detection of any reaction, using an assay system, indicates that an interleukin-18 protein or polypeptide or an interleukin-18α protein or polypeptide is present in the sample. Such techniques permit detection of interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide in a sample of the following tissue or body fluids: blood, spinal fluid, sputum, pleural fluids, urine, bronchial alveolor lavage, lymph nodes, bone marrow, or other biopsied materials.

In one embodiment, the assay system has a sandwich or competitive format. Examples of suitable assays include an enzyme-linked immunosorbent assay, a radioimmunoassay, a gel diffusion precipitin reaction assay, an immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay, and an immunoelectrophoresis assay.

The invention also provides a method for detecting interleukin-18 or interleukin-18α using a DNA molecule which encodes interleukin-18 or interleukin-18α as a probe in a nucleic acid hybridization assay. The probes can be

utilized to identify interleukin-18 genes in other species as well as other isoforms of interleukin-18 which may have altered activity. A sample is contacted with the probe. The detection of any reaction, using an assay system, indicates that a nucleic acid encoding interleukin-18 or interleukin-18α or a fragment thereof is present in the sample. The nucleotide sequences of the present invention may be used in any nucleic acid hybridization assay system known in the art, including, but not limited to, Southern blots (Southern, J. Mol. Biol., 98:508 (1975)); Northern blots (Thomas et al., Proc. Nat'l Acad. Sci. USA, 77:5201-05 (1980)); Colony blots (Grunstein et al., Proc. Nat'l Acad. Sci. USA, 72:3961-65 (1975), which are hereby incorporated by reference). Alternatively, the isolated DNA molecules of the present invention can be used in a gene amplification detection procedure (e.g., a polymerase chain reaction). See H.A. Erlich et. al., "Recent Advances in the Polymerase Chain Reaction", Science 252:1643-51 (1991), which is hereby incorporated by reference.

The invention further provides a method for quantitating stress in a mammal. An antibody or binding portion thereof specific to an interleukin-18 protein or polypeptide or an interleukin-18α protein or polypeptide is contacted with a sample of tissue or body fluid of the mammal. The amount of interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide present in the sample, which is indicative of the stress in the mammal, is measured using an assay system.

Another embodiment of the invention is a method for quantitating stress in a mammal using a DNA molecule which encodes interleukin-18 or interleukin-18α as a probe in a nucleic acid hybridization assay. Tissue or body fluid of the mammal is contacted with the probe, and the amount of interleukin-18 mRNA or interleukin-18α mRNA present in the

sample, which is indicative of the stress in the mammal, is measured using an assay system described previously.

High stress levels can be used as an indicator of problems which have not yet had a pathological manifestation. High stress levels would indicate the need to investigate and control the sources of stress, whether biological (i.e. an infection), chemical (i.e. exposure to toxic compounds), psychological, etc. Early recognition of high stress levels could thus prevent or minimize damage.

The invention further provides methods of stimulating a cellular immune response. IGIF is a costimulator of cellular immune response and also acts as an antitumor and antimicrobial agent. Accordingly, IGIF can be administered to a mammal to stimulate the immune response. IGIF may also utilized as a costimulator/adjuvant in vaccine protocols to increase the efficiency of vaccine protocols by nducing cellular immunity. In a preferred embodiment of the invention, rat IGIF is administered to a rat to induce the cellular immune response.

EXAMPLES

Example 1 - Experimental Protocols

All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University Medical College. Male Sprague-Dawley rats weighing 300-500 g from Charles River Breeding Laboratories (Boston, MA) were used. For the pharmacological treatment, they received subcutaneous injections of reserpine (Sigma), 10mg/kg in 20% ascorbic acid, or an equivalent volume of the vehicle, 4 h prior to anesthesia and perfusion. Cold stress was given by placing the animals, still in their cage, at 4°C for 4 h. Access to food and water was free, and the light/dark cycle was maintained at 12 h/12 h.

For tissue collection animals were decapitated after treatment and adrenal glands were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C until extraction of RNA.

Differential Display - Differential display was performed as described previously using the RNA image system (GenHunter Corp., Brookline, MA) (Liang, P. et al., Science, 257:967-997 (1992), which is hereby incorporated by reference). In brief, mRNA was extracted using the vehicle-treatment animal dissected 4 h after the injection. The RNA was then treated with RNase-free DNase I (GenHunter Corp.) and reverse-transcribed using the three one-based anchored oligo(dT) strategy (Liang, P. et al., Nucleic Acids Res., 25:5763-5764 (1994), which is hereby incorporated by reference). PCR was performed in the presence of [α-^{35}S]dATP and oligonucleotides specifically designed for differential display (Liang, P. et al., Science, 257:967-997 (1992), which is hereby incorporated by reference). PCR conditions were 40 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 2 min, and extension at 72°C for 30 s; the last extension step at 72°C was prolonged for 5 min. Radiolabeled reaction products were subjected to electrophoresis on a 6% denaturing polyacrylamide/urea gel. The differentially expressed PCR products were excised from the gel, reamplified by PCR and subcloned into the pCR-TRAP cloning vector (GenHunter Corp.). Inserts were sequenced by dideoxynucleotide sequencing.

Cloning and Probe - Rat IGIF was isolated by RT-PCR from the adrenal gland of a reserpine-treated animal. Two μg of total RNA were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT)$_{15}$. The cDNA obtained was amplified with a mouse specific 5'-primer (5'-AACAATGGCTGCCATGTCA-G-3')(SEQ. ID. No. 5) and a rat specific 3'-primer (5'-AGTGAACATTACAGATTTATC-CC-3')(SEQ. ID. No. 6). The

amplification was performed in 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; the last extension step at 72°C was prolonged for 5 min. The amplified cDNA was purified from agarose gel, subcloned in pCR-TRAP cloning vector, and sequenced by the dideoxynucleotide method.

Rat IGIF probe was obtained by PCR using the pCR-TRAP clone containing the subcloned rat IGIF as template. The primers used for the amplifications were the same 3'-primer utilized for the cloning and a new 5'-primer internal to the isolated fragment: 5'-ACTGTACAACCGCAGTAATACGG-3' (SEQ. ID. No. 7). PCR conditions were the same as described above. The amplified fragment (437 bp in length) was purified from agarose gel, labeled with [α-^{35}S]dATP using the random primer method, and used as a probe for *in situ* hybridization.

In Situ Hybridization - *In situ* hybridization was performed as described (Weiser, M. et al., Neurosci., 13:3472-3484 (1993), which is hereby incorporated by reference). In brief, animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrate and 10 units/ml heparin sulfate followed by cold formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The adrenal and pituitary glands were postfixed in the fixative for 1 h and stored in 30% sucrose overnight. Free floating sections (40 μm), obtained on a freezing microtome, were placed in vials containing 2 × SSC (1 × SCC is 0.15 M NaCl and 0.015 M sodium citrate) and 50 mM dithiothreitol. Tissues were prehybridized in 50% formamide, 10% dextran, 2 × SSC, 1 × Denhardt's solution, 10 mM dithiothreitol, and 0.5 mg/ml sonicated and denatured salmon sperm DNA. Denatured [^{35}S]dATP-labeled cDNA probe was added to the vial (10^7 cpm/ml/vial), and hybridization was carried out overnight at 48°C. The sections were washed in serial

dilutions of SSC at 48°C starting with 2 × SSC and ending with 0.1 × SSC. After a 15-min wash in 0.05 M phosphate buffer, sections were mounted and dehydrated. For determination of optimal developing time, slides were exposed to Kodak XAR-5 film at 4°C. Slides were subsequently dipped in Kodak NTB-2 emulsion and exposed at 4°C. After developing in Kodak D-19 developer at 16°C, sections were fixed in Kodak fixer, counterstained with cresyl violet, dehydrated, and coverslipped.

Example 2 - Differential Display and Subcloning

Differential display analysis was performed on mRNA from reserpine and vehicle-treated rat adrenal gland RNA. The samples were collected from two animals 4 h after the injection of reserpine (10 mg/kg) or vehicle solution (20% ascorbic acid), and the reactions were done using different primer combinations as described in Example 1. The reactions that generated PCR products exhibiting a differential profile were repeated, and the band of interest was excised from the gel and reamplified.

A preliminary *in situ* hybridization confirmed the pattern of induction of the isolated molecule and localized the mRNA to the adrenal cortex. The cDNA was then subcloned into pCR-TRAP, single colonies were analyzed for the presence of the insert, and positive clones were sequenced.

Example 3 - IGIF Identification and Isolation

The sequence of the subcloned cDNA, about 200 bp in length, was submitted to GenBank™, EMBL, DDBJ, and PDB data bases for homology comparison and found to have 90% homology to the 3′ end of mouse mRNA for IGIF precursor polypeptide (accession number D49949). The isolated cDNA

corresponded to the last 80 bp of the mouse IGIF coding region and the remaining to the 3'-untranslated region.

Rat IGIF coding region was isolated from the adrenal gland by reverse transcriptase-PCR. Two distinct PCR products were visible: a very abundant band and a faint shorter one. This pattern was reproduced even when a different primer combination was used. Their sequence overlap with that of the fragment derived from differential display so that it was possible to reconstitute the whole sequence (SEQ. ID. No. 1 and 3). They appear to be two different isoforms of the same molecule identified as rat IGIF on the basis of the high homology with mouse IGIF. The longer transcript shows 91% homology to mouse IGIF at both the nucleotide and the amino acid level and encodes a protein of 194 amino acids, the glycine in position 8 and the proline in position 64 are absent in mouse (SEQ. ID. No. 2). The shorter isoform lacks a fragment of 57 bases, a probable exon, and the corresponding 19 amino acids in the predicted peptide (SEQ. ID. No. 4). The presence at the 3' end of exons of the dinucleotide AG ($A^{359}G^{360}$ and $A^{416}G^{417}$) suggests the exon is between bases 361 and 417, although it could also be between bases 359 and 415. The reconstituted frame encodes for Phe^{119}-Glu^{120}-Lys^{121} in both cases.

<u>Example 4</u> - In Situ Hybridization

IGIF mRNA induction was strong and specific in both reserpine-treated and the cold-stressed animals, whereas little or no signal was detected in control or in vehicle-treated animals (Fig. 1, A-E). The induction was localized to the adrenal cortex, specifically to the zona reticularis and fasciculata that synthesize glucocorticoids. No mRNA was detected in the medulla where reserpine is known to act. The level of transcription returned to basal within 24 h of the end of the cold exposure. The presence of IGIF

mRNA was also detected in the posterior lobe of the pituitary gland (Fig. 1*F*) although the differences in the levels of transcripts do not appear significant in the various conditions.

The presence of IGIF mRNA was discovered by differential display in the adrenal gland of reserpine-treated rats. This early evidence was confirmed with the isolation of a rat IGIF by reverse transcriptase-PCR from the adrenal gland of an animal injected with reserpine. Two PCR products were identified. They appear to be two isoforms (IL-18 and IL-18α) of the same molecule, although further characterization might be required to conform it. On the basis of structural homologies observed between mouse IGIF and human interleukins-1 (Bazan, J.F. et al., Nature, 379:591 (1996), which is hereby incorporated by reference), it is curious to notice that the 19 amino acids missing in the short isoform correspond to the amino-terminal peptide encoded by exon 7 of human IL-1α and human IL-1β.

Subsequent *in situ* hybridization analysis then clearly showed the localization of IGIF transcripts in the zona reticularis and fasciculata of the adrenal cortex and confirmed the pattern of induction after reserpine treatment. Reserpine, which causes catecholamine depletion and blocks their reuptake, also induces evident sickness when injected. Since it is known that the pharmacological and the biochemical effects of reserpine on the adrenal glands resemble that of stress (Muéller, R.A. et al., Mol. Pharmacol., 7:463-469 (1969); Joh, T.H. et al., Proc. Natl. Acad. Sci. U.S.A., 70:2767-2771 (1973); Wessel, T.C. et al., Mol. Brain Res., 15:349-360 (1992), which are hereby incorporated by reference), the possibility that IGIF induction was actually due to the stressful condition the treated animals undergo was investigated. *In situ* hybridization analysis was repeated comparing the adrenal

glands of reserpine-treated, vehicle, control, and cold-stressed animals.

Cold stress, given as exposure to 4°C for 4 h prior to anesthesia, is indeed sufficient to stimulate IGIF mRNA transcription in the same areas of the adrenal cortex. The observation that the mRNA levels return to basal within 24 h of the cold stress suggests that IGIF transcription is a rapid response to stress probably accounting for an immediate rather than for an adaptive response. Mouse IGIF is believed to be produced as precursor and secreted after cleavage of the leader peptide at aspartate in amino acid position 35 (26 in rat form) mediated by an interleukin-1β convertase-like enzyme (ICE-like). Interleukin-1β convertase mRNA, and its catalytic activity has been found in adrenal and pituitary glands of rats treated with LPS (Tingsborg, S. et al., Brain Res., 712:153-158 (1996), which is hereby incorporated by reference), and IGIF protein has been detected in the supernatant of cultured Kupffer cells after stimulation with LPS (Okamura, H. et al., Nature, 378:88-91 (1995), which is hereby incorporated by reference). IGIF could be secreted from the adrenal and pituitary glands following a stressful experience and act as neuroimmunomodulator or require the presence of at least a second stimulus, such as an infectious agent.

IGIF mRNA was found in Kupffer cells and macrophages where its level does not appear to change even after stimulation with infectious and inflammatory agents (Okamura, H. et al., Nature, 378:88-91 (1995); Ushio, S. et al., Immunol., 156:4274-4279 (1996), which are hereby incorporated by reference). Here was observed a pattern of IGIF mRNA induction, notably following a stressful experience and, very interestingly, located in the adrenal gland. IGIF and glucocorticoids seems to be both synthesized by the same cells of the adrenal cortex, induced by stress, and yet appear to have different functions on the

immune system. The production by the adrenal gland of molecules with protective effects against stress has long been postulated, and the anti-inflammatory properties of the glucocorticoids are seen as a means to prevent immune system overreaction (Munck, A. et al., Endocr. Rev., 5:25-44 (1984), which is hereby incorporated by reference). The small amount of data available on the biological activity of IGIF shows, on the contrary, its property as proinflammatory agent and stimulator of the cell-mediated immune response, thus its ability to enhance the immune functions in a situation of potential danger. In this view, IGIF may play a critical role in autoimmune diseases.

The exact effects that IGIF would exert on the immune system in this context and its physiological meaning remain to be investigated. Certainly the role of the adrenal gland as effector of immunomodulation needs to be reconsidered and deserves great attention.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these therefore are considered within the scope of the invention as defined in the claims which follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cornell Research Foundation, Inc.

(ii) TITLE OF INVENTION: INTERFERON-GAMMA INDUCING FACTOR IN NEUROENDOCRINE CELLS

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Rochester
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(E) COUNTRY: USA
(F) ZIP: 14603

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/025,141
(B) FILING DATE: 09-SEP-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/043,087
(B) FILING DATE: 08-APR-1997

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(C) REFERENCE/DOCKET NUMBER: 19603/1433

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 716-263-1304
(B) TELEFAX: 716-263-1600

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 722 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTTCACTGTA CAACCGCAGT AATACGGAGC ATAAATGACC AAGTTCTCTT CGTTGACAAA   180
AGAAACCCGC CTGTGTTCGA GGACATGCCT GATATCGACC GAACAGCCAA CGAATCCCAG   240
ACCAGACTGA TAATATATAT GTACAAAGAT AGTGAAGTAA GAGGACTGGC TGTGACCCTA   300
TCTGTGAAGG ATGGAAGGAT GTCTACCCTC TCCTGTAAAA ACAAAATCAT TTCCTTTGAG   360
GAAATGAATC CACCTGAAAA TATTGATGAT ATAAAAAGTG ATCTCATATT CTTTCAGAAA   420
CGTGTGCCAG GACACAACAA AATGGAATTT GAATCTTCCC TGTATGAAGG ACACTTTCTA   480
GCTTGCCAAA AGGAAGATGA TGCTTTCAAA CTCGTTTTGA AAAGGAAGGA TGAAAATGGG   540
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AA                                                                  722
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 194 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Phe Ile Asp Asn Thr Leu Tyr Leu Ile Pro Glu Asp Asn Gly Asp
            20                  25                  30
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35 40 45

Arg Ser Ile Asn Asp Gln Val Leu Phe Val Asp Lys Arg Asn Pro Pro
50 55 60

Val Phe Glu Asp Met Pro Asp Ile Asp Arg Thr Ala Asn Glu Ser Gln
65 70 75 80

Thr Arg Leu Ile Ile Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu
85 90 95

Ala Val Thr Leu Ser Val Lys Asp Gly Arg Met Ser Thr Leu Ser Cys
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Lys Asn Lys Ile Ile Ser Phe Glu Glu Met Asn Pro Pro Glu Asn Ile
115 120 125

Asp Asp Ile Lys Ser Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly
130 135 140

His Asn Lys Met Glu Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu
145 150 155 160

Ala Cys Gln Lys Glu Asp Asp Ala Phe Lys Leu Val Leu Lys Arg Lys
165 170 175

Asp Glu Asn Gly Asp Lys Ser Val Met Phe Thr Leu Thr Asn Leu His
180 185 190

Gln Ser

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 665 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ACCAGACTGA TAATATATAT GTACAAAGAT AGTGAAGTAA GAGGACTGGC TGTGACCCTA 300

TCTGTGAAGG ATGGAAGGAT GTCTACCCTC TCCTGTAAAA ACAAAATCAT TTCCTTTGAG 360

AAACGTGTGC CAGGACACAA CAAAATGGAA TTTGAATCTT CCCTGTATGA AGGACACTTT 420

CTAGCTTGCC AAAAGGAAGA TGATGCTTTC AAACTCGTTT TGAAAAGGAA GGATGAAAAT 480

GGGGATAAAT CTGTAATGTT CACTCTTACT AACTTACATC AAAGTTAGGT ATTAAGGTTT 540

CTGTATTCCA GAAAGACGAT TAGTATACAC GAGCCTTATG ATAACCTACT CTGTATTTCT 600

ATGACAAAAT ACCTGAGGCC GCATGATTTA TAGAGTAAAC AAGCTTGATT GCCCAAAAAA 660

AAAAA 665

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 175 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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35 40 45

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50 55 60

Val Phe Glu Asp Met Pro Asp Ile Asp Arg Thr Ala Asn Glu Ser Gln
65 70 75 80

Thr Arg Leu Ile Ile Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu
85 90 95

Ala Val Thr Leu Ser Val Lys Asp Gly Arg Met Ser Thr Leu Ser Cys
100 105 110

Lys Asn Lys Ile Ile Ser Phe Glu Lys Arg Val Pro Gly His Asn Lys
115 120 125

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145 150 155 160

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTGAACATT ACAGATTTAT CCC 23

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTGTACAAC CGCAGTAATA CGG 23

WHAT IS CLAIMED:

1. An isolated nucleic acid molecule encoding a rat interleukin-18 protein or polypeptide or a rat interleukin-18α protein or polypeptide.

2. The isolated nucleic acid molecule according to claim 1, wherein said nucleic acid encodes rat interleukin-18 protein or polypeptide.

3. The isolated nucleic acid molecule according to claim 2, wherein said nucleic acid molecule encodes a protein or polypeptide having an amino acid sequence as shown in SEQ. ID. No. 2 or is a complement thereof.

4. The isolated nucleic acid molecule according to claim 2, wherein said nucleic acid molecule has a nucleotide sequence of SEQ. ID. No. 1 or a nucleotide sequence which has 95% or greater homology to SEQ. ID. No. 1 or is a complement thereof.

5. The isolated nucleic acid molecule according to claim 1, wherein said nucleic acid encodes a rat interleukin-18α protein or polypeptide.

6. The isolated nucleic acid molecule according to claim 5, wherein said nucleic acid molecule encodes a protein or polypeptide having an amino acid sequence as shown in SEQ. ID. No. 4 or is a complement thereof.

7. The isolated nucleic acid molecule according to claim 5, wherein said nucleic acid molecule has a nucleotide sequence of SEQ. ID. No. 3 or a nucleotide sequence which has 95% or greater homology to SEQ. ID. No. 3 or is a complement thereof.

8. The isolated nucleic acid molecule according to claim 1, wherein said nucleic acid is a deoxyribonucleic acid.

9. The isolated nucleic acid molecule according to claim 8, wherein said deoxyribonucleic acid is cDNA.

10. The isolated nucleic acid molecule according to claim 1, wherein said nucleic acid is a ribonucleic acid.

11. The isolated nucleic acid molecule according to claim 10, wherein said ribonucleic acid is mRNA.

12. An antisense nucleic acid molecule complementary to or capable of hybridizing under stringent conditions to the mRNA according to claim 11.

13. An expression vector comprising the antisense nucleic acid molecule according to claim 12.

14. The antisense nucleic acid molecule according to claim 12, wherein said nucleic acid comprises a ribonucleic acid.

15. An expression vector comprising a heterologous nucleic acid molecule according to claim 1.

16. The expression vector according to claim 15, wherein the nucleic acid molecule is in correct reading frame and proper sense orientation.

17. The expression vector according to claim 15, wherein said expression vector is selected from the group consisting of a plasmid, a virus, and a bacteriophage.

18. A host cell transformed with a heterologous DNA molecule according to claim 1.

19. A host cell according to claim 18, wherein the heterologous DNA molecule is in an expression vector.

20. A method of producing a rat interferon-γ inducing factor, said method comprising:

transforming a cell with the nucleic acid molecule of claim 1 and

causing said cell to express said nucleic acid molecule and to produce a rat interferon-γ inducing factor.

21. An isolated rat interleukin-18 protein or polypeptide or rat interleukin-18α protein or polypeptide.

22. The isolated protein or polypeptide according to claim 21, wherein said protein or polypeptide is a rat interleukin-18 protein or polypeptide.

23. The isolated protein or polypeptide according to claim 22, wherein said protein or polypeptide has an amino acid sequence of SEQ. ID. No. 2.

24. The isolated protein or polypeptide according to claim 21, wherein said protein or polypeptide is a rat interleukin-18α protein or polypeptide.

25. The isolated protein or polypeptide according to claim 24, wherein said protein or polypeptide has an amino acid sequence of SEQ. ID. No. 3.

26. An antibody or binding fragment thereof specific for the isolated protein or polypeptide according to claim 21.

27. The antibody according to claim 26, wherein said antibody or binding fragment thereof is a monoclonal antibody.

28. The antibody according to claim 26, wherein said antibody or binding fragment thereof is a polyclonal antibody.

29. A composition comprising the isolated protein or polypeptide according to claim 21 and a compatible carrier.

30. A method for detection of interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide in a sample of tissue or body fluids comprising:

providing an antibody or binding portion thereof according to claim 26;

contacting the sample with the antibody or binding portion thereof; and

detecting any reaction which indicates that an interleukin-18 protein or polypeptide or an interleukin-18α protein or polypeptide is present in the sample using an assay system.

31. A method according to claim 30, wherein the assay system is selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, a gel diffusion precipitin reaction assay, an immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay, and an immunoelectrophoresis assay.

32. A method for detection of an interleukin-18 protein or polypeptide or an interleukin-18α protein or polypeptide in a sample of tissue or body fluids comprising:

providing a DNA molecule according to claim 1 as a probe in a nucleic acid hybridization assay;

contacting the sample with the probe; and

detecting any reaction which indicates that a nucleic acid molecule which encodes interleukin-18 or interleukin-18α or a fragment thereof is present in the sample.

33. A method for quantitating stress in a mammal comprising:

providing an antibody or binding portion thereof according to claim 26;

contacting tissue or body fluid of the mammal with the antibody or binding portion thereof; and

measuring interleukin-18 protein or polypeptide or interleukin-18α protein or polypeptide present in the tissue or body fluid, which is indicative of the stress in the mammal, using an assay system.

34. A method according to claim 33, wherein the assay system is selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, a gel diffusion precipitin reaction assay, an immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay, and an immunoelectrophoresis assay.

35. A method for quantitating stress in a mammal comprising:

providing a DNA molecule according to claim 1 as a probe in a nucleic acid hybridization assay;

contacting tissue or body fluid of the mammal with the probe; and

measuring interleukin-18 mRNA or interleukin-18α mRNA present in the tissue or body fluid, which is indicative of the stress in the mammal.

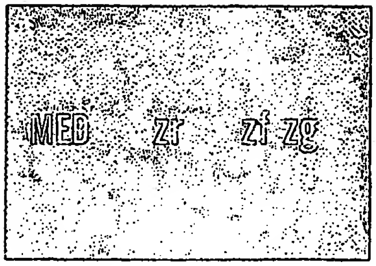

FIG. 1A

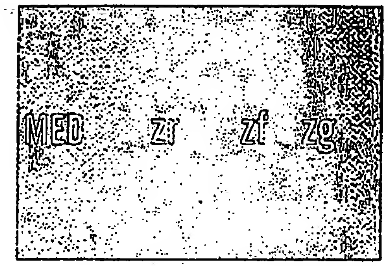

FIG. 1D

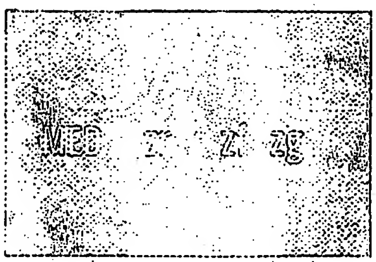

FIG. 1B

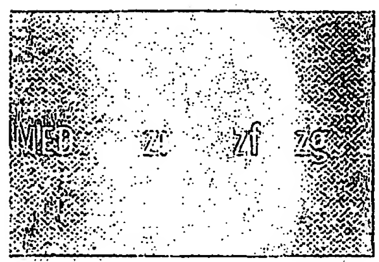

FIG. 1E

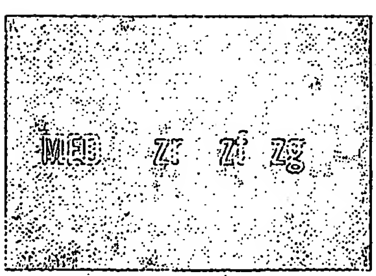

FIG. 1C

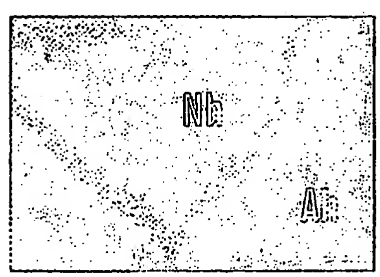

FIG. 1F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15891

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/24; C07K 14/54; A61K 31/20
US CL :530/351; 435/69.52, 320.1, 325; 536/23.5; 424/85.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351; 435/69.52, 320.1, 325; 536/23.5; 424/85.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	USHIO, S., et al. Cloning of the cDNA for Human IFN-γ-Inducing Factor, Expression in *Escherichia coli*, and Studies on the Biologic Activity of the Protein. Journal of Immunology. 1996, Vol. 156, pages 4724-4729, especially the abstract, Figure 1, and the Discussion section at pages 4277-4279.	1-4, 8-11, 15-23, 29 — 5-7, 24-25
Y — A	EP 0 692 536 A2 (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) 17 January 1996, pages 2 and 6, SEQ ID NOs: 4 and 5.	1-4, 8-11, 15-23, 29 — 5-7, 24, 25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 NOVEMBER 1997	02 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CONTI, B. et al. Induction of Interferon-γ Inducing Factor in the Adrenal Cortex. Journal of Biological Chemistry. 24 January 1997, Vol. 272, No. 4, pages 2035-2037, especially the abstract, Figure 1, and the Discussion at page 2037.	1-11, 15-25, 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15891

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-11, 15-25, and 29

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Keyword databases: USPTO - APS, Medline, Biosis, CAS, EPO online, Derwent-WPI
Search terms: IGIF, interferon/IFN (gamma) inducing, IL/interleukin-18
Sequence databases: GenBank/EMBL/DDBJ, GeneSeq, SwissProt, PIR, USPTO issued

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

I. Claims 1-11, 15-25, and 29, drawn to nucleic acids encoding rat IGIFs and associated products, their use to produce recombinant protein, and the protein produced by such methods.

II. Claims 12, 13, and 14, drawn to antisense nucleic acids.

III. Claims 26-28, drawn to antibodies.

IV. Claims 30, 31, 33, and 34, drawn to a binding assay.

V. Claims 32 and 35, drawn to a hybridization assay.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

Pursuant to 37 C.F.R. § 1.475(d), this Authority considers that when multiple products, methods of making such products, and methods of using such products are claimed, the Main Invention shall consist of the first-recited invention and the first-recited inventions of the other categories related thereto. Here, the first invention is the product, an IGIF-encoding nucleic acid, and the first-recited method of using such product is its use in the recombinant production of IGIF polypeptides. No method of making the subject nucleic acids is claimed.

Further pursuant to 37 C.F.R. § 1.475(d), this Authority considers that any feature which the inventions of Groups II-V share with the Main Invention of Group I does not constitute a special technical feature within the meaning of PCT Rule 13.2. Consequently, the products of groups II and III and the methods of groups IV and V are not so linked in any pairing as to form a single general inventive concept within the meaning of PCT Rule 13.1.